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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference Case 21407	**FOR FURTHER ACTION**	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/EP 03/10573	International filing date *(day/month/year)* 23.09.2003	Priority date *(day/month/year)* 27.09.2002
International Patent Classification (IPC) or both national classification and IPC C12N15/81		
Applicant DSM IP ASSETS B.V. et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 4 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the opinion
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☐ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
09.03.2004	20.01.2005
Name and mailing address of the international preliminary examining authority: European Patent Office - P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk - Pays Bas Tel. +31 70 340 - 2040 Tx: 31 651 epo nl Fax: +31 70 340 - 3016	Authorized Officer Aslund, J Telephone No. +31 70 340-4393

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/EP 03/10573

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:

Description, Pages

1-34 as originally filed

Claims, Numbers

1-26 received on 04.08.2004 with letter of 03.08.2004

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☒ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☐ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)).

(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	4-13, 21-25
	No:	Claims	1-3,14-20, 26
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-26
Industrial applicability (IA)	Yes:	Claims	1-26
	No:	Claims	

2. Citations and explanations

see separate sheet

Re Item V

Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

Citations

D1: WO 96 09393 A (REYNOLDS TECHNOLOGIES INC ;BIOSOURCE TECH INC (US); HANLEY KATHLEE) 28 March 1996 (1996-03-28)

D2: ROBINSON G W ET AL: 'CONSERVATION BETWEEN HUMAN AND FUNGAL SQUALENE SYNTHETASES: SIMILATITIES IN STRUCTURE, FUNCTION, AND REGULATION' MOLECULAR AND CELLULAR BIOLOGY, WASHINGTON, DC, US, vol. 13, no. 5, 1 May 1993 (1993-05-01), pages 2706-2717, XP000604626 ISSN: 0270-7306

D3: SHIMADA HIROSHI ET AL: 'Increased carotenoid production by the food yeast Candida utilis through metabolic engineering of the isoprenoid pathway' APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 64, no. 7, July 1998 (1998-07), pages 2676-2680, XP002271872 ISSN: 0099-2240

D4: Genbank Accession number: X99718, 19960915, Corran A.J.

D5: Genbank Accession number: DB84401, 19991120 Kikuti et al.

Novelty - Article 33(2) PCT

The present application does not meet the criteria of Article 33(1) PCT, because the subject-matter of claims 1-3, 14-20,26 is not new in the sense of Article 33(2) PCT.

Claims 1 (e), 2(p), 3 relate to polynucleotides encoding polypeptides of >51.3% sequence identity to Seq Id no 3. D4 discloses a polynucleotide encoding a polypeptide of >52% sequence identity to Seq Id no 3. Consequently said claims lack novelty. Furthermore, claim 2 section (t) is not novel over D5. The following 17 nucleotides of Seq Id no 1 are also found in D5: tccttccttctctctct.

Claim 14 relates to an antisense polynucleotide. Without a definition of size, the claim could relate to any fragment, and consequently the claim lacks novelty. Claims 15-20 dependent on claim 14 are by consequence also not novel.

Claim 26 is not novel over D3 as stated in the previous communication.

Inventive step - Article 33(3) PCT

Proteins in the prior art have around 52% sequence identity to Seq Id no 3. The sought scope of protection is not commensurate with the disclosed subject-matter. Many undisclosed sequences falling under the scope of claims 1, 2 are clearly more related to the prior art sequences than what has been contributed by the subject-matter of the present application.
Furthermore, it appears that the application only provides theorethical examples of increased carotenoid production in Phaffia rhodozyma by antisense inhibition of squalene synthase. Thus, without further supporting data it is impossible to judge whether the application provides a solution to a technical problem or not.

Claims

1. An isolated polynucleotide comprising a nucleic acid molecule one or more selected from the group consisting of

(a) nucleic acid molecules encoding at least the mature form of the polypeptide depicted in SEQ ID NO:3;

(b) nucleic acid molecules comprising the coding sequence as depicted in SEQ ID NO:2;

(c) nucleic acid molecules whose nucleotide sequence is degenerate as a result of the genetic code to a nucleotide sequence of (a) or (b);

(d) nucleic acid molecules encoding a polypeptide derived from the polypeptide encoded by a polynucleotide of (a) to (c) by way of substitution, deletion and/or addition of one or several amino acids of the amino acid sequence of the polypeptide encoded by a nucleotide of (a) to (c);

(e) nucleic acid molecules encoding a polypeptide derived from the polypeptide whose sequence has an identity of 51.3 % or more to the amino acid sequence of the polypeptide encoded by a nucleic acid molecule of (a) or (b);

(f) nucleic acid molecules comprising a fragment encoded by a nucleic acid molecule of any one of (a) to (e) and having squalene synthase activity;

(g) nucleic acid molecules comprising a polynucleotide having a sequence of a nucleic acid molecule amplified from a *Phaffia* nucleic acid library using the primers depicted in SEQ ID NO:4, 5, and 6;

(h) nucleic acid molecules encoding a polypeptide having squalene synthase activity, wherein said polypeptide is a fragment of a polypeptide encoded by any one of (a) to (g);

(i) nucleic acid molecules comprising at least 15 nucleotides of a polynucleotide of any one of (a) to (d);

(j) nucleic acid molecules encoding a polypeptide having squalene synthase activity, wherein said polypeptide is recognized by antibodies that have been raised against a polypeptide encoded by a nucleic acid molecule of any one of (a) to (h);

(k) nucleic acid molecules obtainable by screening an appropriate library under stringent conditions with a probe having the sequence of the nucleic acid molecule of any one of (a) to (j), and encoding a polypeptide having squalene synthase activity;

(l) nucleic acid molecules whose complementary strand hybridizes under stringent conditions with a nucleic acid molecule of any one of (a) to (k), and encoding a polypeptide having squalene synthase activity.

2. An isolated polynucleotide comprising a nucleic acid molecule one or more selected from the group consisting of:

(m) nucleic acid molecules comprising the nucleotide sequence as depicted in SEQ ID NO:1;

(n) nucleic acid molecules whose nucleotide sequence is degenerate as a result of the genetic code to a nucleotide sequence of (m);

(o) nucleic acid molecules encoding a polypeptide derived from the polypeptide encoded by a polynucleotide of (m) or (n) by way of substitution, deletion and/or addition of one or several amino acids of the amino acid sequence of the polypeptide encoded by a nucleotide of (m) or (n);

(p) nucleic acid molecules encoding a polypeptide derived from the polypeptide whose sequence has an identity of 51.3 % or more to the amino acid sequence of the polypeptide encoded by a nucleic acid molecule of (m);

(q) nucleic acid molecules comprising a fragment encoded by a nucleic acid molecule of any one of (m) to (p) and having squalene synthase activity;

(r) nucleic acid molecules comprising a polynucleotide having a sequence of a nucleic acid molecule amplified from a *Phaffia* nucleic acid library using the primers depicted in SEQ ID NO:4, 5, and 6;

(s) nucleic acid molecules encoding a polypeptide having squalene synthase activity, wherein said polypeptide is a fragment of a polypeptide encoded by any one of (m) to (r);

(t) nucleic acid molecules comprising at least 15 nucleotides of a polynucleotide of any one of (m) to (o);

(u) nucleic acid molecules encoding a polypeptide having squalene synthase activity, wherein said polypeptide is recognized by antibodies that have been raised against a polypeptide encoded by a nucleic acid molecule of any one of (m) to (s);

(v) nucleic acid molecules obtainable by screening an appropriate library under stringent conditions with a probe having the sequence of the nucleic acid molecule of any one of (m) to (u), and encoding a polypeptide having squalene synthase activity;

(w) nucleic acid molecules whose complementary strand hybridizes under stringent conditions with a nucleic acid molecule of any one of (m) to (v), and encoding a polypeptide having squalene synthase activity.

3. The isolated polynucleotide of claim 1 or 2, wherein said polynucleotide encodes an amino acid sequence which is identified by SEQ ID NO:3 or has an identity of 51.3 % or more with SEQ ID NO:3.

4. The isolated polynucleotide of any one of claims 1 to 3, wherein said polynucleotide is derived from a strain of *Phaffia rhodozyma* or *Xanthophylomyces dendrorhous*.

5. A method for making a recombinant vector comprising inserting the polynucleotide of any one of claims 1 to 4 into a vector.

6. A recombinant vector containing the polynucleotide of any one of claims 1 to 4 or produced by the method of claim 5.

7. The vector of claim 6 in which the polynucleotide of any one of claims 1 to 4 is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic cells.

8. A method of making a recombinant organism comprising introducing the vector of claim 6 or 7 into a host organism.

9. The method of claim 8, wherein said host organism is selected from *E. coli*, baculovirus, or *S. cerevisiae*.

10. The recombinant organism containing the vector of claim 6 or 7, or produced by the method of claim 8 or 9.

11. A process for producing a polypeptide having squalene synthase activity comprising culturing the recombinant organism of claim 10 and recovering the polypeptide from the culture of said recombinant organism.

12. A polypeptide obtainable by the process of claim 11.

13. An antibody that binds specifically to the polypeptide of claim 12.

14. An antisense polynucleotide against the polynucleotide of any one of claims 1 to 4.

15. A method for making a recombinant vector comprising inserting the polynucleotide of claim 14 into a vector.

16. A recombinant vector containing the polynucleotide of claim 14 or produced by the method of claim 15.

17. The vector of claim 16 in which the polynucleotide of claim 14 is operatively linked to expression control sequences allowing expression in prokaryotic or eukaryotic cells.

18. A method of making a recombinant organism comprising introducing the vector of claim 16 or 17 into a host organism.

19. The method of claim 18, wherein said host organism belongs to a strain of *Phaffia rhodozyma* or *Xanthophylomyces dendrorhous*.

20. The recombinant organism containing the vector of claim 16 or 17, or produced by the method of claim 18 or 19.

21. The recombinant organism of claim 20, wherein said organism is characterized in that whose gene expression of squalene synthase is reduced compared to the host organism, thereby is capable of producing carotenoids in an enhanced level relative to a host organism.

22. The recombinant organism according to claim 21, wherein the gene expression of squalene synthase is reduced by means of the technique selected from antisense technology, site-directed mutagenesis, error prone PCR, or chemical mutagenesis.

23. A process for producing carotenoids, which comprises cultivating the recombinant organism of claim 21.

24. The process of claim 23, wherein said carotenoids are selected one or more from astaxanthin, β-carotene, lycopene, zeaxanthin, canthaxanthin.

25. The process according to claim 23, wherein the gene expression of squalene synthase is reduced in the recombinant organism of claim 21 by means of the technique selected from antisense technology, site-directed mutagenesis, error prone PCR, or chemical mutagenesis.

26. A process for the production of a carotenoid by culturing a microorganism under suitable conditions and, optionally, recovering the resulting carotenoid, wherein the microorganism is characterized in that its gene expression of squalene synthase is reduced, e.g. by means of the technique selected from antisense technology, site-directed mutagenesis, error prone PCR, or chemical mutagenesis.

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